doi:10.1016/j.jmb.2011.04.045 J. Mol. Biol. (2011) 409, 826–838

Contents lists available at www.sciencedirect.com

Journal of Molecular Biology

journal homepage: http://ees.elsevier.com.jmb

Membrane Integrity and Amyloid Cytotoxicity: A Model Study Involving Mitochondria and Lysozyme Fibrillation Products

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Received 26 January 2011; received in revised form 12 April 2011; accepted 17 April 2011
Available online 4 May 2011

Edited by S. Radford

Keywords:
hen egg white lysozyme; mitochondria; membrane permeabilization; protofibrils; cytotoxicity

Recent findings implicate that fibrillation products, the protein aggregates formed during the various steps leading to formation of mature fibrils, induce neurotoxicity predominantly in their intermediate oligomeric state. This has been shown to occur by increasing membrane permeability, eventually leading to cell death. Despite accumulating reports describing mechanisms of membrane permeabilization by oligomers in model membranes, studies directly targeted at characterizing the events occurring in biological membranes are rare. In the present report, we describe interaction of the original native structure, prefibrils and fibrils of hen egg white lysozyme (HEWL) with mitochondrial membranes, as an *in vitro* biological model, with the aim of gaining insight into possible mechanism of cytotoxicity at the membrane level. These structures were first characterized using a range of techniques, including fluorescence, size-exclusion chromatography, dynamic light scattering, transmission electron microscopy, dot blot analysis and circular dichroism. HEWL oligomers were found to be flexible/hydrophobic structures with the capacity to interact with mitochondrial membranes. Possible permeabilization of mitochondria was explored utilizing sensitive fluorometric and luminometric assays. Results presented demonstrate release of mitochondrial enzymes upon exposure to HEWL oligomers, but not native enzyme monomer or mature fibrils, in a concentration-dependent manner. Release of cytochrome *c* was also observed, as reported earlier, and membrane stabilization promoted by addition of calcium prevented release. Moreover, the oligomer–membrane interaction was influenced by high concentrations of NaCl and spermine. The observed release of proteins from mitochondria is suggested to occur by a nonspecific perturbation mechanism.

Introduction

It is now generally accepted that protein misfolding leading to aggregation is a key pathogenic feature of various amyloid-related disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, type II diabetes and prion diseases.[1,2] Ordered protein aggregates, often referred to as amyloid fibrils, are commonly found in these diseases. Moreover, recent findings have indicated that the ability to form amyloid fibrils is

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Abbreviations used: HEWL, hen egg white lysozyme; ThT, thioflavin T; ANS, 1-anilino-naphthalene 8-sulfonate; SEC, size-exclusion chromatography; DLS, dynamic light scattering; TEM, transmission electron microscopy; MDH, malate dehydrogenase; GDH, glutamate dehydrogenase; CS, citrate synthase; AK, adenylate kinase; MAO, monoamine oxidase; EDTA, ethylenediaminetetraacetic acid.

0022-2836/$ - see front matter © 2011 Published by Elsevier Ltd.

a generic property of all proteins rather than a characteristic feature of only those associated with pathological conditions.[1–5] Formation of amyloid fibrils by various proteins and polypeptides precedes by the occurrence of metastable, partially folded oligomeric intermediates often referred to as protofibrils,[6–8] which have been found to be cytotoxic.[2,9–12] Moreover, the toxic activity of prefibrillar oligomers may be due to the fact that they share a common structure, suggesting that they may also share a common mechanism of toxicity.[13] The fact that different amyloids arise from either the cytosolic or the extracellular protein points to the plasma membrane as a potential primary target that is accessible to both compartments.[2,11,14,15] Although there appears to be a consensus on membrane permeabilization by amyloid oligomers, it is disputed whether this is due to the discrete channel formation or to a nonspecific perturbation of bilayer integrity, since evidence for both possibilities has been obtained.[14–20] In addition to plasma membrane as a primary target, internal organelles, such as mitochondria, may also be affected.[21] Recent reports indicate that mitochondrial dysfunction may play a critical role in the development of neurodegeneration in a number of pathological conditions.[22,23] Accumulation of β-peptide and α-synuclein has been shown to occur with predominant localization in the inner-membrane structures.[24,25] Of the various consequential events, cell death is often attributed to an increase in mitochondrial membrane permeability.[26,27] Lysozyme, more specifically hen egg white lysozyme (HEWL), is one of the most extensively studied proteins whose structure and physiochemical properties have been well characterized.[28] With a high p*I* of 11,[29] the protein bears a net positive charge over a broad pH range and has a high affinity for anionic and neutral phospholipids, both at low and high ionic strengths.[30] In addition to its lipid-binding properties, it was found to induce release of the aqueous contents of uncharged vesicles, presumably as a result of its insertion into the lipid bilayer, causing disruption.[30,31] HEWL has been shown to abundantly form well-defined amyloid fibrils under *in vitro* conditions, making it an ideal model for the study of amyloid aggregation.[32] Additionally, it is homologous to human lysozyme, the variants of which have been shown to form amyloid fibrils, implicated in hereditary systemic amyloidosis.[33] Although a large body of evidence suggests membrane perturbation as a primary mechanism of toxicity in neurodegenerative diseases, most of such conclusions have been based on studies involving phospholipid model systems. In the present study, mitochondria isolated from rat brain were used as an *in vitro* model to examine the possible destructive effects of HEWL oligomers and fibrils. It is proposed that the organelle with its well-characterized membranes consisting of various biologically active components, combined with its exceptional biochemical composition and compartmental diversity, may provide an extremely useful model system for biophysical studies related to mechanism of cytotoxicity at the membrane level, leading to cell death.

Results and Discussion

Membrane disruption by amyloid oligomers is often considered as a primary mechanism of toxicity in neurodegenerative disorders, but the mechanism by which these structures eventually cause cell dysfunction and death is not clearly understood. One of the problems associated with these investigations is related to the fact that the oligomeric species are often unstable, making detailed structural analyses difficult.[34] It is generally believed that enhancement of hydrophobicity initiated by protein misfolding provides these structures with the ability to interact with membranes, resulting in loss of membrane integrity and thereby cytotoxicity.[9,35–38] Furthermore, such hydrophobicity-based toxicity mechanism has been shown to be shared by bacterial toxins and viral proteins.[39] In addition to hydrophobic interactions, electrostatic forces involving charged residues in protein structures and charged or polar lipid molecules have been concluded to provide the second major interactions in the association process.[40]

Two mechanisms of membrane disruption have been recently proposed. The first argues that membrane permeabilization is induced by formation of specific, discrete ion channels that may be inhibited by specific channel blockers,[18–20] and the second suggests involvement of a nonspecific mechanism of membrane perturbation in the absence of unitary conductance.[14–17] HEWL is one of the best-characterized proteins whose amyloidogenesis, in particular, has been extensively studied under *in vitro* conditions.[32,41–43] In the present study, interaction of HEWL oligomers and fibrils on mitochondrial membranes was investigated. Oligomeric and not fibrillar structures have been found to have the capacity to cause release of mitochondrial enzymes.

Oligomer characterization

The structural and morphological features of HEWL oligomers have been investigated by employment of a range of techniques, including fluorescence [(thioflavin T (ThT), 1-anilino-naphthalene 8-sulfonate (ANS) and acrylamide quenching)], size-exclusion chromatography (SEC), dynamic light scattering (DLS), transmission electron microscopy (TEM), circular dichroism (CD) and dot blot